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Barbara Thalenfeld
New York
Nov. 1982

Molecular Cloning

A LABORATORY MANUAL

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Cloning in Plasmids

In principle, cloning in plasmid vectors is very straightforward. The plasmid DNA is cleaved with a restriction endonuclease and joined in vitro to foreign DNA. The resulting recombinant plasmids are then used to transform bacteria. In practice, however, the plasmid vector must be carefully chosen to minimize the effort required to identify and characterize recombinants. The major difficulty is to distinguish between plasmids that contain sequences of foreign DNA and vector DNA molecules that have recircularized without insertion of foreign sequences. Recircularization of the plasmid can be limited to some extent by adjusting the concentrations of the foreign DNA and vector DNA during the ligation reaction. However, a number of procedures, described below, have been developed either to reduce recircularization of the plasmid still further or to distinguish recombinants from nonrecombinants by genetic techniques.

Insertional Inactivation

This method can be used with plasmids that carry two or more antibiotic-resistance markers (see Fig. 1.1). The DNA to be inserted and the purified plasmid DNA are digested with a restriction enzyme that, in this example,

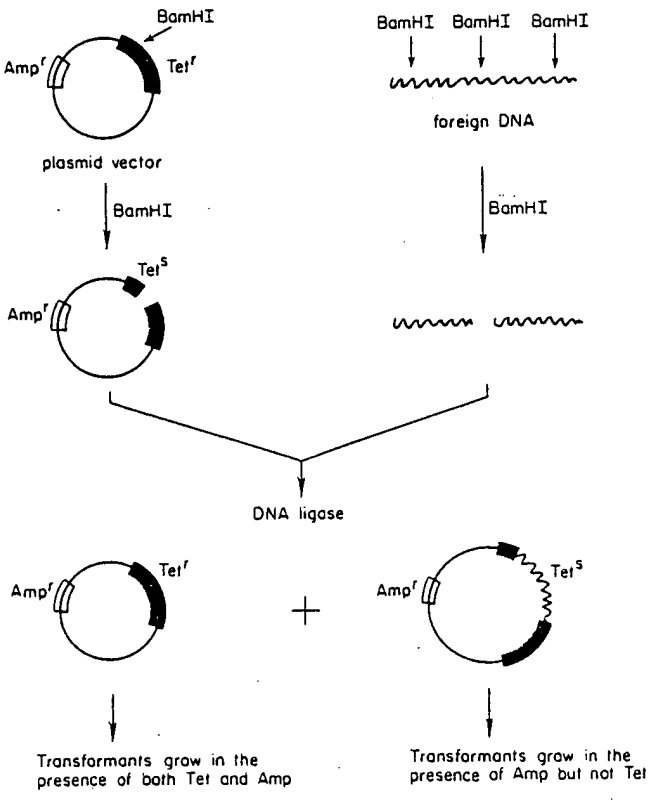


Figure 1.1

Insertional inactivation.

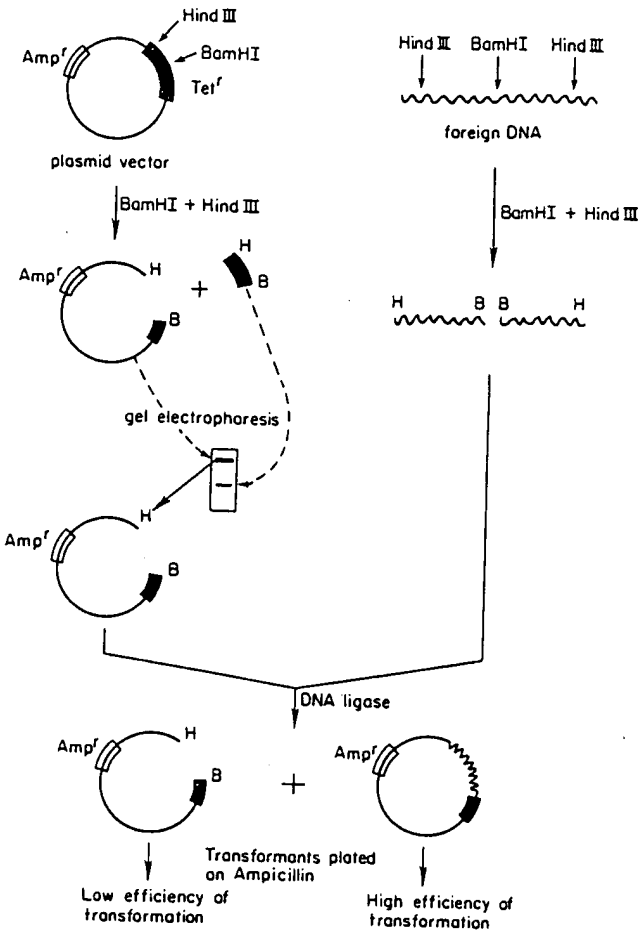


Figure 1.3

Directional cloning.

foreign DNA segment with 5′-terminal phosphates can be ligated efficiently to the dephosphorylated plasmid DNA to give an open circular molecule containing two nicks (see Fig. 1.4). Because circular DNA (even nicked circular DNA) transforms much more efficiently than linear plasmid DNA, most of the transformants will contain recombinant plasmids. A protocol for phosphatase treatment of plasmid DNA is given on page 133.

Problems in Cloning Large DNA Fragments in Plasmids

Finally, the size of the foreign DNA to be inserted can also affect the ratio of transformants containing recombinant plasmids to those containing recircularized vectors. In general, the larger the insertion of foreign DNA, the lower the efficiency of transformation. Thus, when cloning large DNA fragments (>10 kb), it is especially important to take all possible measures to keep the number of recircularized vector molecules to a minimum. Even so, the background is relatively high, and it is usually necessary to use an in situ hybridization procedure (Grunstein and Hogness 1975; Hanahan and Meselson 1980) to identify recombinant transformants.

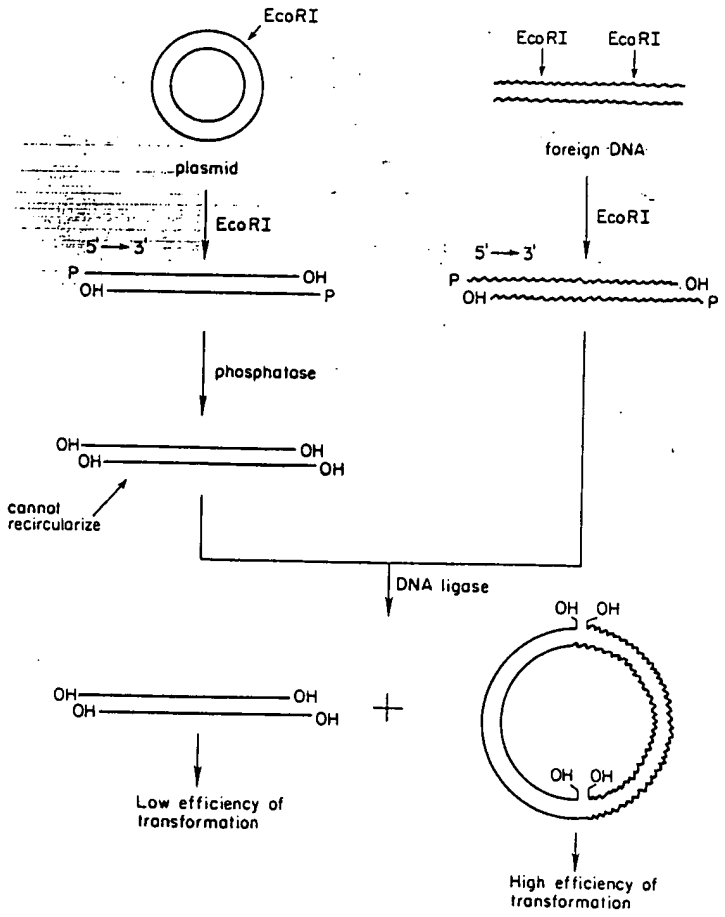


Figure 1.4

Use of phosphatase to prevent recircularization of vector DNA.

The second problem can be minimized by selecting fragments of eukaryotic DNA of a certain size (30–45 kb) for ligation to the cosmid vector. Insertion of two or more such fragments into the same cosmid will result in a molecule too large to be packaged into a bacteriophage λ particle.

Recently, Ish-Horowicz and Burke (1981) have described another method for cosmid cloning that overcomes the first two of these problems. In this procedure, which is designed for the vector pJB8 and illustrated in Figure 1.10, the cosmid vector is divided into two aliquots, each of which is cleaved with a different restriction enzyme cutting either to one side or the other of the *cos* sequence. The resulting full-length, linear DNAs are dephosphorylated with alkaline phosphatase. It is this dephosphorylation that prevents formation of tandem vectors and suppresses the background of bacterial colonies containing cosmids lacking inserts. The dephosphorylated DNAs are then both cleaved with *Bam*HI and mixed to generate cohesive ends, which can be ligated to eukaryotic DNA prepared by partial cleavage with *Mbo*I or *Sau*3A followed by dephosphorylation. This protocol gives only one

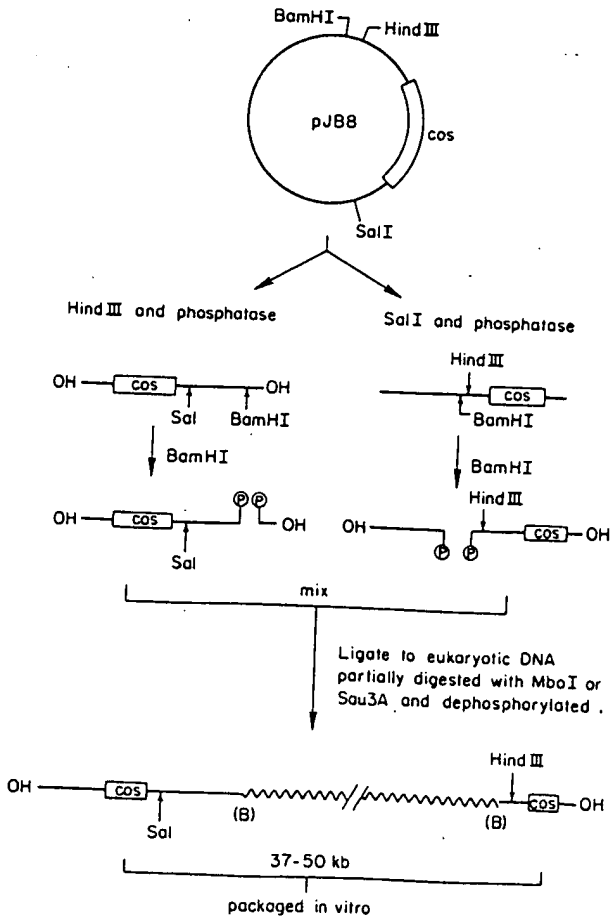


Figure 1.10

Efficient cloning in cosmids (Ish-Horowicz and Burke 1981).

10× nick-translation buffer	5 μl
DNA	1 μg
unlabeled dNTPs (if needed)	1 nmole of each (1 μl of a 1 mM solution)
[α-^{32}P]dNTPs	100 pmoles
H_2O	to 44 μl

Chill the mixture to 0°C. Make a 10^4-fold dilution of a small quantity of a stock solution of DNase (1 mg/ml) in ice-cold, nick-translation buffer containing 50% glycerol. The diluted enzyme is stable when stored at −20°C in this buffer (see page 451).

5. Add 0.5 μl of diluted DNase I (0.1 μg/ml) to the reaction mixture. Mix by vortexing.

6. Add 5 units (as defined by Richardson et al. 1964) of *E. coli* DNA polymerase I. Mix.

7. Incubate at 16°C for 60 minutes.

   Note. If the reaction is carried out at higher temperature, a considerable amount of "snapback" DNA may be generated by DNA polymerase copying the newly synthesized strand.

9. Stop the reaction by adding 2 μl of 0.5 M EDTA.

10. Using the DE-81 binding or TCA precipitation assays described on page 473, determine the proportion of [α-^{32}P]dNTPs that have been incorporated into DNA.

11. Separate the nick-translated DNA from unincorporated dNTPs either by chromatography on or centrifugation through a small column of Sephadex G-50 (see pages 464–467).

Notes

i. The specific activity of the nick-translated DNA depends not only on the specific activity of the dNTPs, but also on the extent of nucleotide replacement of the template. This can be controlled by varying the amount of DNase I in the reaction. The aim is to establish conditions that will result in incorporation of about 30% of the [α-^{32}P]dNTPs into DNA.

It is possible to obtain more extensive labeling by a replacement reaction, in which the 3′ exonuclease activity of the enzyme first digests duplex DNA to produce molecules with recessed 3′ termini. On subsequent addition of labeled dNTPs, the partially digested DNA molecules serve as primer · templates that are regenerated by the polymerase into intact, double-stranded DNA. Molecules labeled to high specific activity by this technique are used chiefly as hybridization probes. They have two advantages over probes prepared by nick-translation. First, they lack the artifactual hairpin structures that can be produced during nick translation. Second, they can easily be converted into strand-specific probes by cleavage with suitable restriction endonucleases (see Fig. 4.1).

However, this method, by contrast to nick translation, does not produce a uniform distribution of label along the length of the DNA. Furthermore, the 3′ exonuclease activity degrades single-stranded DNA much faster than double-stranded DNA, so that after a molecule has been digested to its midpoint, it will dissociate into two, half-length, single strands that will be rapidly degraded. It is therefore important to stop the exonuclease reaction before the enzyme reaches the center of the molecule. Consequently, the replacement synthesis method yields a population of molecules that are fully labeled at their ends but that contain progressively decreasing quantities of label toward their centers. Thus, the size of the smallest restriction fragment in a mixture of fragments dictates the maximum extent to which all the fragments can be labeled.

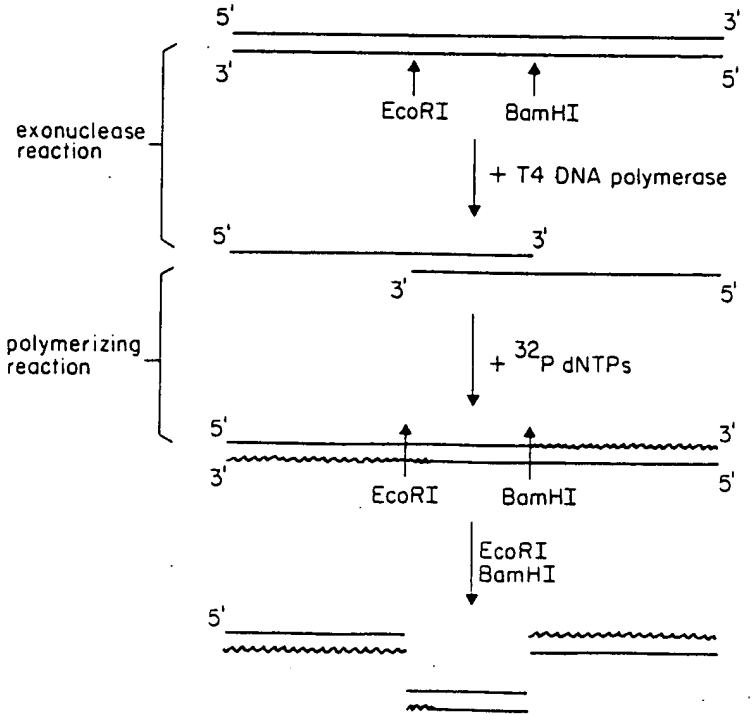


Figure 4.1

Preparation of Hybridization Probes Using Reverse Transcriptase and Random Primers

This is the method of choice for synthesizing ^{32}P-labeled probes of high specific activity from single-stranded DNA or from RNA templates.

1. Mix 1.0 μg of template (linear double-stranded or single-stranded DNA or RNA) with 20 μl of water. Heat to 100°C in a boiling-water bath for 5 minutes. Chill quickly in ice water.

2. Add:

calf thymus or salmon sperm primer (50 mg/ml)	10 μl
5× random-primer buffer	20 μl
2 mM solution of each unlabeled dNTP	2 μl
[α-^{32}P]dNTP (sp. act. > 400 Ci/mM)	250 pmoles (100 μCi)
reverse transcriptase	200 units
H_2O	to 100 μl

5× Random-primer buffer

0.25 M Tris · Cl (pH 8.1)
10 mM dithiothreitol
25 mM $MgCl_2$
0.2 M KCl

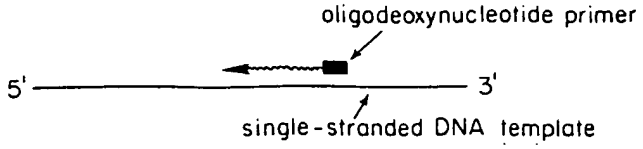
3. Mix and incubate at 37°C for 1 hour.

4. Add 2 μl of 0.5 M EDTA. Separate the labeled DNA from unincorporated dNTPs either by chromatography through a column of Sephadex G-50 or by spun-column chromatography (see pages 464–467). Approximately 30% of the [α-^{32}P]dNTP should have been incorporated into DNA.

Notes

i. RNA templates can be removed at the end of the reaction as follows: After addition of EDTA (step 4), add 12 μl of 3 M NaOH and incubate for 12 hours at 37°C. The alkaline solution can then be applied directly to Sephadex G-50 equilibrated in TE (pH 7.6).

ii. Hybridization probes made by reverse transcriptase from DNA are single-stranded copies of the templates:



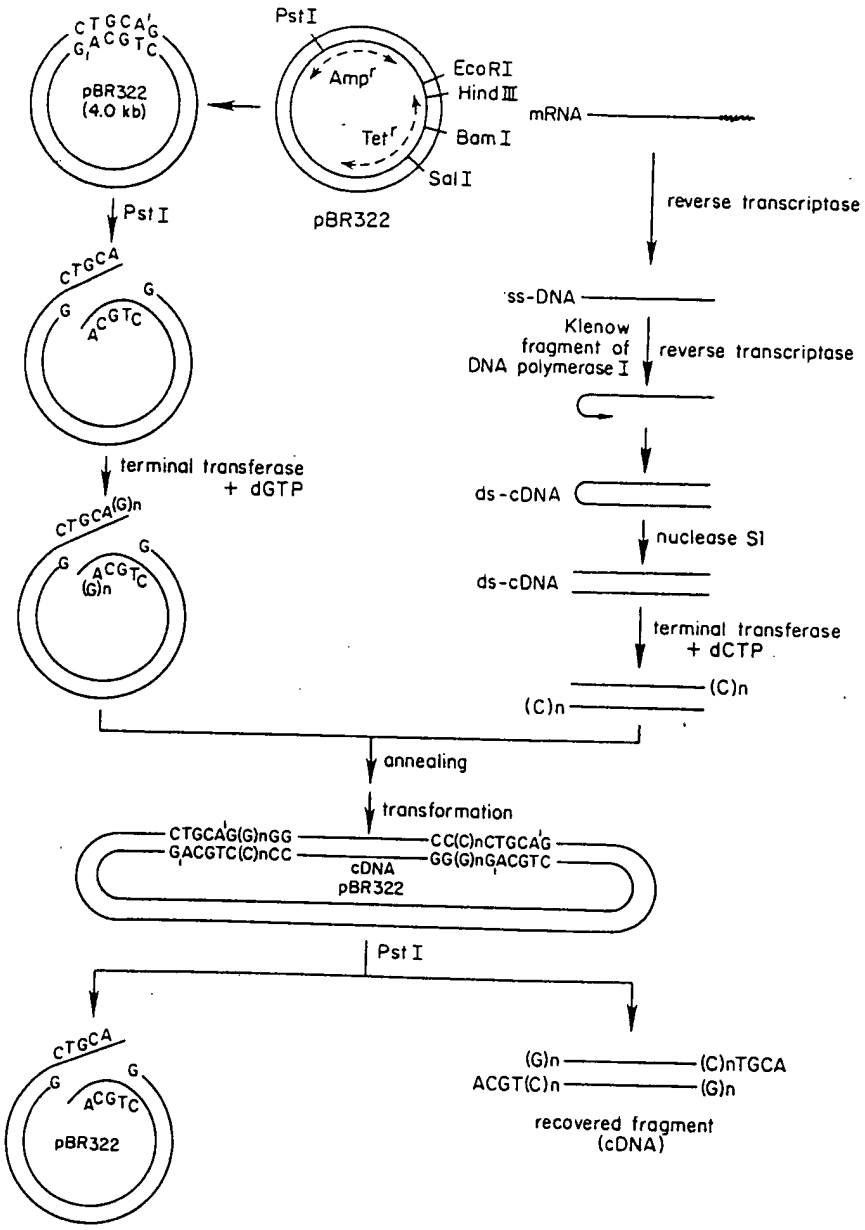


Figure 7.1

The conditions first used to achieve full-length, second-strand cDNA synthesis by DNA polymerase I (Efstratiadis et al. 1976) are still widely used (Wickens et al. 1978). In brief, the reaction is carried out at pH 6.9 to minimize the 5′ → 3′ exonuclease activity of DNA polymerase I and at 15°C to minimize the possibility of synthesizing "snapback" DNA. The Klenow fragment of DNA polymerase I, which lacks the 5′ → 3′ exonuclease activity, has also been successfully employed to synthesize the second cDNA strand.

Okayama and Berg find that full-length or nearly full length cDNA copies are preferentially converted to duplex cDNA, and an efficiency of approximately 100,000 transformants per microgram of starting mRNA is obtained. The preferential cloning of long cDNA transcripts is thought to be a consequence of the preferential utilization of full-length reverse transcription by terminal transferase. They speculate that shortened or truncated cDNA strands in the mRNA · DNA duplex are not efficiently recognized by the terminal transferase and are therefore selected against. Although the rabbit α- and β-globin mRNA was used to establish this cDNA cloning procedure, Okayama and Berg indicate that other cDNA clones representing both rare and long (6500-nucleotide) mRNAs have been obtained with this procedure.

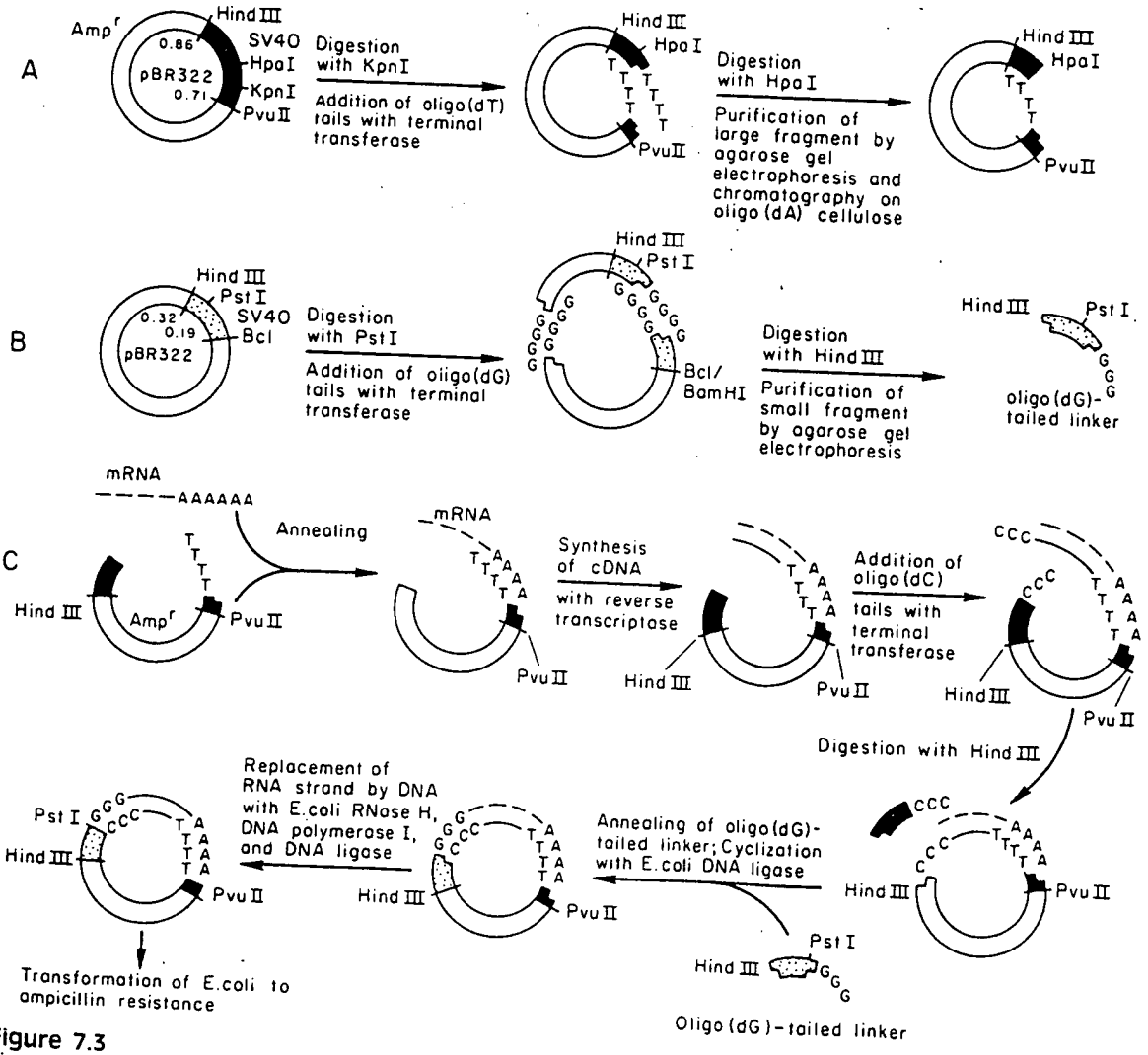


Figure 7.3

Preparation of (*A*) plasmid primer and (*B*) oligo(dG)-tailed linker DNA. (*C*) Steps in the construction of plasmid-cDNA recombinants. pBR322 DNA is represented by the open sections of each ring; SV40 DNA is indicated by the darkened or stippled segments. The numbers next to the restriction site designations are the corresponding SV40 DNA map coordinates.